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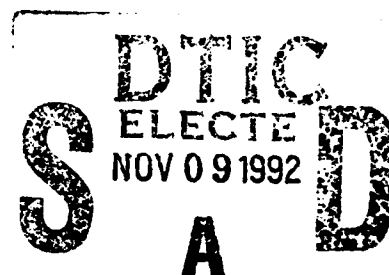
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FINAL TECHNICAL REPORT

February 1, 1989 - July 31, 1992

"Evaluation of Dried Storage of Platelets and RBC for Transfusion:
Lyophilization and other Dehydration Techniques"

Grant No. N00014-89-J-1712
From the Office of Naval Research:
Navy Medical Research and Development Command
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Respectfully submitted, November 2, 1992.

This document has been approved
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INTRODUCTION

This project was put forth to meet the needs expressed by the Naval Medical Research and Development Command for long-term dried storage of human blood cells for transfusion support of combat casualty care. Our main focus was on preparation of platelets for successful dehydration, storage, and rehydration without substantial compromise of hemostatic function. The primary technology was developed as an extension of a pre-existing protocol for drying fixed platelets as a diagnostic product, but many alternative approaches were also tried on an empirical basis. None of these methods had been previously applied to the development of a transfusion product.

A grant was awarded through the Office of Naval Research in the amount of \$851,069 for the performance of this project. Three research laboratories collaborated in this effort: East Carolina University School of Medicine (Principal Investigator = Arthur P. Bode, Ph.D.), The University of North Carolina at Chapel Hill (Principal Investigator = Marjorie S. Read, Ph.D.), and the American Red Cross Mid-Atlantic Regional Blood Services Research Laboratory (Principal Investigator = Stein Holme, Ph.D.). The administrative and fiscal responsibility was centered at E.C.U. and the other two performance sites subcontracted. The activity period covered by the grant was February 1, 1989 through January 31, 1992, with a no-cost extension to July 31, 1992.

There were six Specific Aims proposed and tested under this project; in brief they were:

- (1) To optimize dehydration techniques;
- (2) To analyze cell functionality in vitro;
- (3) To test rehydrated platelets in circulation in animal models;
- (4) To evaluate different conditions for dried storage of platelets;
- (5) To perform hemostasis studies in animals, and;
- (6) To perform cell survival studies in man.

All but the last Aim (cell survival studies in man) were completed for dehydrated platelets. Dried erythrocytes were prepared and evaluated in the above protocols as a secondary effort except for Aim No. 6.

The overall result of this research has been the development of at least two successful protocols for preparation of dried platelets which maintain cell integrity and function when rehydrated. A patent application (serial no. 07/891,277) was filed on May 29, 1992, in the U.S. Patent and Trademark Office to protect the contemplated commercialization of this technology. A licensing agreement is now in preparation with Rhone-Poulenc Rorer, Inc., and Armour Pharmaceutical Company at the date of this report. We hope that the continuation of these efforts will lead to the eventual manufacture of an FDA-approved medical preparation for human transfusion.

Processing of Blood:

The freeze-dried platelets developed in this project were

prepared from several different sources: fresh whole blood from human volunteer donors, citrated platelet concentrates from the clinical blood bank, or fresh whole blood from pigs and dogs at the Francis Owen Blood Research Laboratory in Chapel Hill. The animal platelets were processed specifically for the purpose of autologous infusion studies to test rehydrated platelets' function and survival in circulation. As much or more emphasis was given to the preparation of human platelets in terms of specific protocol development and in vitro analysis.

Whole blood (50-500 mL) collected in citrate anticoagulant (CPDA-1 or ACD formulae) was centrifuged to prepare platelet-rich plasma (PRP) at 22°C. Chilled blood (<18°C) was found unacceptable because of poor platelet yields. In some experiments, the platelet activation inhibitor Prostaglandin E-1 was added to the PRP in 300 nM final concentration. This addition produced better morphology and higher platelet yields in the remaining steps before lyophilization, but it was not judged to be absolutely necessary for the success of the preparation. The platelets were centrifuged out of PRP and resuspended 2-3 times in buffer to wash away extraneous plasma proteins. Various buffers were found to be acceptable; including, saline with trisodium citrate as buffer (pH = 6.5), or saline with di/tri basic phosphate as buffer (pH = 6.5) containing 0.1% (w/v) albumin with or without 1.9 mM theophylline to inhibit platelet activation. As above for PGE-1, the inclusion of theophylline in these washes improved the morphology and yield of platelets slightly prior to lyophilization, but this may not be practical nor necessary in large-scale pharmaceutical preparations.

After washing, the platelets were stabilized for lyophilization by a number of techniques. Techniques using ethanol or high salt were abandoned after limited trials. The use of microwave irradiation at 600 watts for 5-10 seconds with human platelets produced preparations which did not maintain good in vitro function or morphology after lyophilization. Although this technique is attractive because it is non-invasive and non-toxic, further development was deferred in order to pursue the more promising results with preparations lightly fixed with aldehyde cross-linking agents. Water replacement molecules, such as trehalose or sucrose at concentrations up to 1 M, did not suffice to stabilize platelets for lyophilization unless combined with aldehydes.

Three different cross-linking compounds were investigated in this project to stabilize platelets prior to lyophilization. Treatment of platelets with 0.5 - 1.0% glutaraldehyde for 30 - 60 minutes preserved their structural integrity during lyophilization, but the rehydrated platelets showed very little functionality in vitro or in canine cell survival studies. The most successful platelet preparations thus far have been stabilized with 1.8-2.0 % paraformaldehyde for 45-60 minutes at 22°C before lyophilization. An alternative protocol has been developed using 0.01 g/dL permanganate (KMnO_4 or NaMnO_4) for 10 minutes at 22°C which is similar to the "para-platelet" preparation described above by in vitro tests, but has not yet been fully evaluated in vivo.

These two methods differ in the chemistry of fixation; paraformaldehyde is an efficient cross-linker of proteins but weak with membrane lipids, permanganate is a very efficient cross-linker of proteins or lipids and is fast-acting. More parallel testing will be required to compare the attributes and effects of "para-platelets" versus "perm-platelets" in vitro and in vivo.

After a light fixative, the platelets are washed 3x in buffer containing 0.1% (w/v) albumin and brought up in a final resuspension for lyophilization. A high concentration of albumin (5% w/v) or trehalose (250 mM - 1 M) is required in the final suspension prior to freeze-drying to ensure platelet yield and integrity upon rehydration. We have tried many different reagents and concentrations in this step with the typical result being <50% recovery of structurally or functionally intact platelets post-rehydration; the presence of albumin or trehalose raises this yield to 90-100%.

Lyophilization and Storage:

Platelet and red cell preparations were frozen at -20 to -70°C after stabilization and then dried under vacuum in a Thermo-Vac lyophilizer (replaced in the third year of this project with a Virtus 600). Typically, the platelets were dried in 1 - 2 mL aliquots at 0.8 - 1.0 $\times 10^9$ /mL in glass or plastic scintillation vials with screw caps. This small size aliquot was useful in preparing samples for later analysis without having to reconstitute the whole preparation. In the animal models, a single large vial was used to devote the whole preparation to a single experiment. Platelet preparations containing a high concentration of trehalose (250 mM - 1 M) required 3 - 5 days in the lyophilizer to dry; the endpoint was chosen to be when the powder in each drying vial appeared to be loose instead of gelled together. Para-platelets required only 2 - 3 days to dry without trehalose. The percent water remaining in dried platelet preparations has not yet been determined.

Development and testing of stabilization procedures proceeded throughout the project period. Therefore, a product representing ideal processing parameters has not yet been evaluated for integrity after long-term storage. Anecdotal information was gathered on at least three different storage environments for dried platelets during refining of the preparation protocols: storage at -70°C, 4°C in desiccator, room temperature (18 - 26°C) in desiccator. The longest period any preparation was stored before use was two months and no deterioration of surface receptors or function was noted. A formal storage study was begun in June, 1992, and will continue during the renewal project period to compare performance of aliquots of a single para-platelet preparation distributed into all three storage environments. Our preliminary evaluations would suggest a shelf-life of at least four months with acceptable integrity of platelet morphology and structure.

Reconstitution of dried platelet suspensions has been achieved with a variety of buffers and solutions: imidazole pH 7.3 - 7.5

without saline, Tris pH 7.4 without saline, phosphate pH 7.0 - 7.5 with electrolytes or Unisol (an artificial medium optimized for liquid storage of platelet concentrates [see Holme et al., Br. J. Haematol. 66:233-238, 1987]) all seemed to work equally well in rehydrating cell membranes without osmotic shock or lysis. We have recently become aware of a DOD program called REFLUPS for development and testing of resuscitation fluids. Future testing should employ solutions approved in REFLUPS.

Tests and Assays:

Rehydrated platelet preparations were analyzed with a battery of in vitro tests and assays to assess structural integrity and metabolic responsiveness. This battery included: yields (platelet counts during preparation and after rehydration), morphology by light microscopy and electron microscopy, released LDH as a marker or lysis upon reconstitution, aggregation response (OD tracings and counts of single platelets remaining in the supernate), shape change and spreading analysis, procoagulant activity expression, flow cytometry of surface glycoprotein receptors on platelets (GPIb, IIbIIIa), light scatter profile, agglutination of platelets through von Willebrands Factor, and response to hypotonic shock. At the UNC-Chapel Hill performance site, in vivo studies also were carried out to examine adhesion of labelled rehydrated platelets to vessel strips in a Baumgartner perfusion chamber, involvement of platelets in bleeding time wounds and in correction of abnormal bleeding times in von Willebrand's pigs, normal thrombus formation with a surgical open-chest wound in dogs, autologous circulatory lifespan determinations in dogs and pigs, and involvement of rehydrated platelets in fibrin clot formation.

Not all of these tests and assays could be performed on each batch of animal or human platelet preparations. The results section that follows shows representative analyses. Reproducibility between batches has not yet been determined, but will be when the final process parameters are established. The data generated for each batch usually included yield, some assessment of morphology, and either an in vivo experiment or an extensive in vitro workup.

RESULTS:

The product of this research has been documented thus far in the technical reports to ONR, three abstracts at international scientific conferences (see below), two poster/verbal presentations to the Armed services at the invitation of the ONR, and a patent application filed in the U.S. Patent Office. Manuscripts were delayed from submission for publication while the patent description was being prepared, but now two are in final draft. Attached below are the three published abstracts, two tables from the patent application, and data summaries from the other performance sites.

Presented by poster at FASEB meetings, 1990. Federation Proceedings 4:4436, 1990.

STUDIES WITH DRIED AND REHYDRATED PLATELETS FOR TRANSFUSION PRODUCTS. M.S. Read*, A.P. Bode* and R.L. Reddick*, Dept. of Pathology, *University of North Carolina, Chapel Hill, NC 27599, *East Carolina University, Greenville, NC 27858.

Dried-rehydrated platelets are being studied for use as a substitute for fresh platelets in transfusion. Washed platelets from three species (human, canine and porcine) were treated with aldehydes prior to freezing and drying. The rehydrated platelets were compared to fresh platelets for adhesiveness and spread characteristics, ex vivo. The ability of the rehydrated platelets to circulate in vivo was also studied. Canine platelets were stabilized with paraformaldehyde (para) and glutaraldehyde (glut) and dried. Dried rehydrated platelets were layered on formvar-coated grids and observed by scanning electron microscopy. Rehydrated platelets were compared to fresh platelets for morphologic evidence of pseudopod formation and platelet spread characteristics. Both fresh and rehydrated para-treated canine platelets had multiple pseudopodia and normal spread configurations. Glut-treated platelets had fewer pseudopods and few spread platelets were seen. In an ex vivo annular perfusion chamber, porcine and human, but not canine, fresh and para-treated platelets adhere at high shear rates in near equal numbers to exposed subendothelium. The ability of rehydrated canine platelets to remain in circulation in vivo was studied using rehydrated para-treated canine platelets labeled with a fluorescent dye (Synaxis, PKH26-GL) infused into a dog. Blood samples were collected at various time intervals for 2 hours following platelet infusion. Smears made from whole blood and platelet-rich plasma aliquots were examined for fluorescence. Labeled para-treated platelets were found in the blood samples for up to 2 hours post infusion. These studies suggest that fixed, rehydrated platelets can be prepared which will adhere to vascular subendothelium, and remain in the circulation for a period of time sufficient to aid in hemostasis (Aided by ONR N00014-89-J-1712).

Presented at AABB meetings, 1990. AABB Book of Abstracts p. 137, 1990.

STABILIZATION OF PLATELET MEMBRANES FOR LYOPHILIZATION AND DRIED STORAGE. A.P. Bode, M.S. Read, and R.L. Reddick. East Carolina University School of Medicine, and University of North Carolina School of Medicine, Chapel Hill, NC.

Historical attempts to arrest hemorrhage with lyophilized platelet preparations failed because of cellular disruption. Dried membranes are subject to lateral phase separation during dehydration and rehydration unless somehow stabilized. We have used microwave heating, water-replacing molecules, or short incubations with paraformaldehyde (PF) to prevent lysis of lyophilized platelets. Cell integrity was assessed by flow cytometry light scatter and surface antigen analysis, electron microscopy (EM), agglutination with ristocetin or botrocetin, and adhesion studies in a Baumgartner perfusion chamber. Results with PF-treated platelets were superior to those from other stabilization techniques. PF treatment for as short as 35 minutes protected platelets from significant loss of GPIb or GPIIb/IIIa during lyophilization and stabilized the light scatter distribution. Approximately 70% of the rehydrated PF treated platelets retained normal levels of surface GPIb and 80% retained normal levels of GPIIb/IIIa. These preparations showed also relatively intact cell morphology by EM, and rapid agglutination with ristocetin or botrocetin, but showed little or no response in the hypotonic shock test. The most striking property of successfully stabilized lyophilized platelets was their ability to adhere to subendothelium in a Baumgartner chamber and to interact with fibrin in clot formation. These results indicate that maintenance of the hemostatic properties of lyophilized platelets is feasible. Studies to assess the in vivo behavior of these platelets are underway.

Presented at FASEB meetings, 1991. Federation Proceedings 5:3093, 1991.

REHYDRATED PLATELETS MAINTAIN HEMOSTATIC PROPERTIES. M.S. Read, R.L. Reddick and A.P. Bode. University North Carolina School of Medicine, Chapel Hill, NC 27599-7525 and East Carolina University School of Medicine, Greenville, NC 27858-4354.

Freshly prepared platelet concentrates have a short shelf life with rapid loss of function. Platelets have been prepared that can be stored in a dry state, and upon rehydration, retain some hemostatic functions. Paraformaldehyde (PF), microwave radiation (MWR), and trehalose (TL) have been used to stabilize human, canine and porcine platelets prior to drying. Electron microscopy (EM) of stabilized platelets showed poor preservation with MWR and TL. With PF, the platelets had excellent morphology. Agglutination of stabilized platelets with botrocetin or ristocetin is rapid. Recalcification of stabilized platelets in plasma produced platelet-fibrin aggregates and by EM, fibrin fibrils were present throughout the clot and fibrin was in close association to the platelet surface. Using an ex vivo perfusion technique, PF stabilized platelets attached to the subendothelium and attached platelet numbers did not differ significantly from nonfixed controls. The results indicate that rehydrated human and animal platelets stabilized for short times in PF, retain the capability to form platelet-fibrin clots, agglutinate with botrocetin and ristocetin, and adhere to the subendothelium in an ex vivo perfusion system. Stabilization with MWR and trehalose produced platelets which behaved like non-fixed platelets in some agglutination and metabolic tests. Aided by Naval Rsch Grant N-00014-89-J-1712.

Table 1
Performance of Rehydrated Para-Platelets in vitro

<u>Aggregation Studies</u>	<u>Percent Platelets Remaining Unaggregated</u>
1.5 mg/mL ristocetin	12-15% (strong response)
10 μ M ADP	42-85% (weak response)
8 μ g/mL collagen	25-60% (medium response)
<u>Flow Cytometry Studies</u>	<u>Percent Platelets with Normal Immuno-Fluorescence</u>
GPIb (AN-51, SZ-2, SZ-1, MoAbs	90-97% (Equivalent to fresh platelets)
GPIIbIIIa (10E5 Moab)	98-99% (Equivalent to fresh platelets)

The microparticle count after rehydration of platelet preparations prepared as described herein was from 4.5 to 5.0 X 10⁶/mL. The hypotonic shock test response for platelets prepared as described in this Example was 0.030 - 0.036 OD/min (0.100 - 0.150 for fresh platelets). Released lactate dehydrogenase (LDH), the amount of LDH in the supernatant after resuspension of 10⁹ platelets in 1 mL of solution, was from 50 to 200 IU/L (>150 indicates significant cytoplasmic leakage). These results represent the range of findings obtained with human platelets stabilized with 2% paraformaldehyde prior to freeze-drying (approximately 20 preparations).

Table 2

Performance of Rehydrated Perm-Platelets in vitro

<u>Aggregation Studies</u>	<u>Percent Platelets Remaining Unaggregated</u>
1.5 mg/mL ristocetin	15-32% (strong response)
10 μ M ADP	27-42% (medium response)
8 μ g/mL collagen	27-50% (medium response)
<u>Flow Cytometry Studies</u>	<u>Percent Platelets with Normal Immuno-Fluorescence</u>
GPIb (AN-51, SZ-2, SZ-1, MoAbs	91-99% (Equivalent to fresh platelets)
GPIIbIIIa (10E5 Moab)	95-99% (Equivalent to fresh platelets)

Microparticle count after rehydration of platelet preparations fixed by the permanganate process described in this example was $2.6 - 4.0 \times 10^6$ /mL. The hypotonic shock test response for platelets prepared by this process was 0 - 0.030 OD/min (0.100 - 0.150 for fresh platelets). Released LDH was 50 - 200 UI/L. These results represent the range of findings obtained with human platelets stabilized with 0.01 - 0.02% permanganate prior to freeze-drying (approximately 15 preparations).

Final Report on ONR Grant No. N00014-J-89-1712
Submitted September 24, 1992, to Arthur P. Bode, Ph.D.
Principal Investigator, East Carolina University.

Marjorie S. Read, Ph.D., Principal Investigator
Robert L. Reddick, M.D., Co-Principal Investigator
University of North Carolina at Chapel Hill

BACKGROUND:

The overall objective was to prepare dried blood cells that could be rehydrated and used as a transfusion product. Our goal was to remove water from the cell and stabilize the dehydrated cell to withstand freezing, drying and rehydration while retaining biological function.

There were 7 specific aims:

1. To develop methods for removal of water from platelets and red blood cells.
2. To develop the best stabilization procedures for platelets and red blood cells.
3. To establish rehydration procedures.
4. To perform in vitro hemostatic tests on rehydrated cells.
5. To test for the ability of rehydrated cells to circulation.
6. To test in vivo hemostatic ability of rehydrated cells.
7. To estimate cell survival in vivo.

It has been clearly demonstrated that platelets are a primary hemostatic cell and that platelets are active in both the thrombolytic and hemostatic process. Previous experience has shown that platelets can be dried and rehydrated and still retain the ability to agglutinate. Our attention, therefore, was focused on the platelet for the major portion of this work.

We developed protocols for the dehydration, lyophilization, and rehydration of platelets isolated from canine, porcine, and human blood anticoagulated with ACD. In this report, I will list some of the studies and briefly comment on some of the results.

Specific Aims 1, 2, and 3 were addressed during years one and two, and aims 4-7 were addressed during years two and three. A large part of the extension period was devoted to applying the technology for drying platelets to drying red blood cells.

All of these aims were addressed and studied. Specific aim 7 was studied with only fluorescence labelled platelets. We had hoped to be able to establish the survival time with chemically labeled platelets and avoid the used of radioisotopes. In hind site, fluorescence labeling may not be the best method and our circulation data may not be a true reflection of the survival time of rehydrated platelets.

RESULTS:

Specific aim 1: Previous studies with lyophilized platelets have shown that ACD anticoagulant, a citrate wash, and resuspension in either Tris or imidazole buffered-saline provides the best platelets for lyophilization. Room temperature preparations are superior to chilled preparations. Canine platelets were used to study the effects of reducing the water content of platelets prior to freezing. Treatment of cells with increasing salt concentration or diluted ethanol in the wash solutions is an effective means of reducing water content. Morphologic examination of cells treated

with salt, or alcohols, however, revealed activated cells and disturbances of internal structure. The addition of forskolin or prostacyclin did not significantly improve the preparations. It became apparent that removing or reducing the water content was more damaging to the platelets than freezing if freezing was done rapidly in the presence of high concentrations of albumin.

Specific aim 2: In addition to removal of water, the cell membrane must be stabilized or "hardened" prior to freezing and lyophilization. We wanted to find a method of stabilizing the membrane to the extent of protecting the cell from fracture, but not at the cost of "killing" the cell. We were looking for a non-invasive technique, and if possible, a non-chemical stabilizer. Microwave irradiation (Gary R. Login and Ann M. Dvorak, Am. J. Pathol., 1985, 120:230-243) has been used to "fix" tissues for study by electron microscopy (EM). Proper microwave (MW) "fixation" yielded tissues equal to or in some cases superior to those fixed by standard aldehyde treatment. We were able to MW fix platelets which survived freezing and thawing, but not lyophilization. MW fixation of platelets was accomplished in a small home style 600 watt, Sharp Carousel microwave oven, model IIR-4P50. Preparations were microwave heated for seven seconds (raising the temperature to 48-52°C) and immediately cooled with cold buffer, centrifuged, resuspended in 5% albumin, either bovine or canine, and tested. These platelets had excellent botrocetin, and ristocetin agglutination response (5-7 seconds agglutination time) and also retained adenosine diphosphate (ADP) induced aggregation (10-15 seconds). Samples that were treated as above and frozen and thawed likewise agglutinated well. However, samples treated as above but frozen and lyophilized failed to agglutinate with any agents tested.

The addition of various sugars, sucrose, manose and trehalose, in concentrations up to 1.0 M did not significantly enhance the platelet preparations either alone or in conjunction with MW treatment.

By EM examination, MW treated platelets that were lyophilized and rehydrated were swollen, spherical, and had released all contents. Although we feel that this is a method deserving of more research, we felt compelled to move on.

Platelets treated with glutaraldehyde and paraformaldehyde retain good morphology following drying and rehydration. Paraformaldehyde concentrations of 1-2% incubated with platelets for 45-120 minutes, and glutaraldehyde at 0.05-2% for 10 minutes have been tested. Each preparation had some good points, but platelets treated with 1.8% paraformaldehyde for 1-2 hours produced platelets that were the most responsive to agglutinating agents. The aldehyde was removed by washing and the stabilized platelets were frozen at -70°C in a solution containing 5% bovine serum albumin. Lyophilization was overnight in a Virtis Model 600 SL lyophilizer. The dried platelets are stored frozen -20°C or -70°C.

Specific aim 3: Many different rehydration methods were tried, including the simple addition of water, buffers, salts, buffered salts, balanced salt solutions, solutions with and without proteins, and multiple combinations of pH and temperature. The best preparations were seen when platelets were removed from the freezer, allowed to come to room temperature, and rehydrated with room temperature water or buffer, at pH 7.35.

Specific aims 3-7 will be addressed together:

Following rehydration, platelets size as volume distribution, as measured by coulter, were similar to those of fresh platelets. Morphology as assessed by EM and phase were also about the same as washed fresh platelets. Both fresh washed and rehydrated platelets had randomly distributed organelles and some pseudopods, but most retained disk form or shape. When tested with botrocetin or ristocetin, rehydrated platelets agglutinate as well as fresh washed platelets. There was little response to adenosine diphosphate and thrombin.

The ability of rehydrated platelets to adhere to vessel subendothelium was tested in the Baumgartner chamber. Porcine vessels were collected and used fresh or within one week of harvesting. Canine, porcine and human rehydrated platelets were tested. Citrated whole blood of each species was run as a control. Blood with rehydrated platelets was prepared for each species by removing the fresh platelets and substituting rehydrated platelets. Blood was pumped across the everted vessel segments and the cells examined by scanning electron microscopy (SEM). There was no difference in the carpet of platelets seen adherent to the subendothelium with rehydrated and fresh platelets.

SEM of everted vessels from the Baumgartner chamber and grids coated with platelets showed that rehydrated platelets form pseudopods and spread on surfaces like fresh platelets.

Procoagulant activity was determined by the ability of rehydrated platelets to shorten the thrombin clotting time of platelet poor plasma to same degree as fresh platelets. The formation of fibrin and the association of fibrin strands to the platelets in the formed clots was examined by EM. Fibrin was associated with rehydrated platelets in thrombin clots and the platelets were degranulated. These data show that rehydrated platelets prepared as above support clot formation and bind fibrinogen (fibrin).

In vivo studies:

We have studied five normal dogs, two bleeder dogs, three bleeder pigs, one irradiated pig, and one normal pig. We infused rehydrated platelets into dogs and pigs and studied the bleeding time correction, circulation time, participation of rehydrated platelets in thrombus formation, adhesion of rehydrated platelets to injured vessel wall, and adhesion of rehydrated platelets to cut wound sites. In all these studies, rehydrated platelets behaved similarly to fresh platelets. In most, but not all experiments, the bleeding time was corrected. We have been unable to clearly

demonstrate how bleeding time is corrected, but we suspect that it is related to the binding of von Willebrand factor to the rehydrated platelets. Binding of fibrinogen to rehydrated platelets has been shown in vitro and probably plays a role in bleeding time correction with rehydrated platelets. Flow cytometric studies conducted in Dr. Bode's laboratory clearly demonstrate the presence of 80% or greater of the GPIb and GPIb/IIa receptors present. Agglutination studies show GPIb to be functional and labeled anti-fibrinogen antibodies demonstrate fibrinogen on the surface of agglutinated rehydrated platelets.

In areas of vessel wall injury, rehydrated platelets were present either adherent to the wall, media of the vessel or in small clumps within the wound area or lumen. Fluorescent microscopy demonstrates the presence of both single and clumped rehydrated platelets in bleeding time wounds, in the flowing blood and on the cut surface. Light and fluorescent microscopy of fibrin clots removed from the cut ear wound area demonstrates rehydrated platelets present.

We have used PKH-26 to study rehydrated platelet circulation in the dog. Most of the dog platelet infusions have been non survival experiments in which we performed the open chest thrombosis experiments discussed above. Consequently, the time of the experiment has been 2-4 hours.

Three dogs have been used to collect platelet survival data and to study long-term circulation of the rehydrated platelets. In all three dogs we were able to detect labeled rehydrated cells by fluorescent microscopy. However, we have been unable to analyze the data due to conflicting results. It appears that we will be unable to determine half life data with this label in rehydrated platelets. We have seen rehydrated labeled platelets circulate for 24 hours or more, but the numbers were insufficient to obtain half-life. We suspect that quenching of the label is responsible for low yields and inconsistent results.

Studies completed during the 1992 extension period:

Studies of antibody response to multiple infusions of rehydrated platelets are being conducted in dogs. The study is designed to show response of the dogs to repeated infusions of autologous or homologous rehydrated platelets and platelets from heterologous donors. Serum is collected and tested for presence of platelet antibodies at designated times following platelet infusion. These studies are underway, but early data has not shown any antibody production. These early data are not conclusive since we are not half way through the protocol.

In progress are studies of the surface antigens, fibrinogen, VWF, PDGF, factor XIII, and fibrinonectin. We are examining the binding of antigen to the surface of rehydrated platelets and to rehydrated platelets stimulated with botrocetin, thrombin, and ADP.

We also used the extension period to apply the protocols developed for drying platelets to the drying of canine red blood cells (RBC). RBC fixed with glutaraldehyde were morphologically superior to those stabilized with paraformaldehyde. There were few

crenated cells and no red cell ghosts when glutaraldehyde was used. Dried RBC rehydrated in phosphate buffer was chosen as the best preparation. Some of the preparations released hemoglobin upon rehydration, but most do not. Tests of RBC for ability to bind oxygen have indicated that rehydrated RBC have a higher affinity for oxygen than fresh RBC. In tests designed to measure oxygen saturation, rehydrated RBC showed a 50% saturation (P50) of 5.7 - 6.1. Fresh RBC have a P50 of 30.1.


We have infused three dogs with rehydrated RBC. One animal received between 1 and 2% of his total RBC volume of rehydrated fluorescent labeled rehydrated RBC. Blood was drawn and labeled rehydrated RBC on a blood smear were counted. Based on the blood volume, original RBC count and the number of rehydrated cells given, we expected to see 4 labeled cells per field. The smear from blood drawn immediately post infusion, contained 4 labeled RBC. Thirty minute post infusion blood smear contained 3 labeled cells, the 2 hour post infusion smear had 2-3 labeled cells, the 24 hour post infusion had 2 labeled cells, the 48 hour sample showed no labeled rehydrated RBC on the smears. The animal showed no adverse effects and is well one month post treatment. A second animal was infused with both labeled rehydrated platelets and labeled rehydrated RBC. The platelet volume was 10-15% of original platelet volume and the rehydrated RBC volume was 1-3% of original fresh RBC count. The experiment was designed to study participation of rehydrated platelets and rehydrated RBC in induced thrombus and induced clot formation in an anesthetized dog. The time frame for the experiment was about 2 hours. Following infusion, whole blood was collected immediately post infusion, 30 minutes and at 2 hours. Blood clots were collected and examined for labeled RBC and platelets. The clots were harvested from chest wound, ear bleeding time cut, and from chest stab wound. At the end of the experiment the animal was sacrificed. Both labeled rehydrated platelets and RBC circulated throughout the experiment. Rehydrated RBC (and rehydrated platelets) were seen in clots from the ear and chest as well as in flowing blood from the cut ear. A third infusion of a 50% volume of rehydrated RBC was not successful and resulted in death of the animal. Necropsy data and data from blood and tissue samples are being studied. The effects of rehydrated cells on clotting, the effect of the increased volume of cells, and the rehydrated preparation are being analyzed.

We will continue to study the effects of drying on hemoglobin binding and release. We are pursuing a collaboration with Dr. John Parker on rehydrated RBC transport and calcium-sodium exchange in the dog.

REPORT ON THE INVESTIGATION OF LYOPHILIZED CELLS

STUDY CONDUCTED BY
RESEARCH LABORATORY OF ARC MID-ATLANTIC REGION
NORFOLK VA

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Report prepared by 
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OBJECTIVE

The role of the Research laboratory of ARC Mid-Atlantic Region in this project was to evaluate the morphologic, biochemical and functional integrity of lyophilized frozen platelets and red cells by performing a wide variety of assays.

MATERIALS AND METHODS

Platelet preparation for in vitro testing:

All liquid samples were thawed. 1 mL of the platelet storage medium UNISOL (Holme et al., Brit J Haematol 1987;66:233) or TRIS buffer was added for resuspension. The additive solution-suspended platelets were washed once and resuspended in autologous CPD-plasma or in the respective additive solutions for in vitro testing for in vitro testing.

In vitro assays:

Platelet count and size distribution were performed using a Coulter counter as described previously (Holme et al., Vox Sang 1990;59:12).

Platelet morphology evaluation and scoring were performed using a phase microscope as described by Kunicki et al., Transfusion 1975;14:414.

Platelet shape change and hypotonic shock response were tested in a Chronolog Aggregometer as described previously (Holme et al., Vox Sang 1990;59:12). Platelet shape change was quantitated by changes in light transmission.

Red blood cell preparation for in vitro testing:

All of the samples were thawed and 1 ml of the storage medium Unisol (see above) was added to the lyophilized samples for resuspension.

In vitro assays:

Morphology evaluation was performed by examination of the samples under the microscope. 100 RBC were classified as either ghost or intact. Intact cells were further classified as spheres or discoid.

Deformability by filtration studies.

This assay was performed by filtering a 5 ml RBC sample through a 20 μ m pore filter (Millipore) with determination of the filtration time.

RESULTS AND DISCUSSION

The results described below are presented according to the method used in preparation of the lyophilized cells.

LYOPHILIZED PLATELETS.

1. Use of 1 M trehalose or sucrose with 1mg/mL PGE₁.

The platelet samples were thawed aliquots taken for microscopic examination. Morphologically, all the platelets were spherical with dendrites. Also, platelet fragments were observed. The samples were then centrifuged and resuspended in Unisol. Of the human platelets, 50 % were spherical; and 50 % dendritic. Clumping and fragmentation with debris were present. Of the canine platelets, 40 % to 50 % were spheres, 30 % were dendrite, and 20 % to 30 % were balloons. Functional testing of these platelets in Unisol showed no response to hypotonic shock or platelet shape change with ADP. There was no difference with platelets processed with trehalose or sucrose. It was concluded that the platelets in these samples had lost their morphologic and functional integrity and were not suitable for transfusion and that trehalose, sucrose, and PGE₁ did not provide any protective effect.

2. Fixation in paraformaldehyde for 30-60 minutes.

The platelet samples were spun and resuspended in TRIS buffer at a pH of 7.4. Microscopically, all the platelets were intact, no debris or fragmentation was seen. 80 % were spherical in shape, 20 % had irregular shape with dendrites. Functional testing was performed using hypotonic shock response and extent of shape change with no response. There was no effect of fixation time or the concentration of paraformaldehyde used. It was concluded that the platelets in these samples had some morphologic integrity intact; however, the functional integrity appeared to be lost.

3. Fixation in various concentrations of KMnO₄ with trehalose, human serum albumin (HSA), and sucrose.

After thawing and resuspension in UNISOL, the platelets in all samples fixed with KMnO₄ showed marked clumping with few, less than 10 %, discoid platelets. The presence of trehalose appeared to have a positive effect in that less clumping was observed and there were fewer spheres and ballooned shaped platelets. However, there was no functional response to hypotonic shock and shape change with ADP after resuspension in CPD-plasma. It was concluded that the platelets in these samples had some morphologic integrity intact, especially when trehalose was used as a protective agent; however, the functional integrity appeared to be lost.

4. Fixation in a low concentration (0.01 %) of KMnO₄ with trehalose/TRIS, human serum albumin (HSA).

After thawing and resuspension in UNISOL, the samples showed that 30-50 % of the platelets had normal discoid morphology. Size distribution also demonstrated normal distribution indicating maintenance of morphologic integrity with no fragmentation or swollen (balloon)

platelets. However, the platelets showed no functional response with hypotonic shock and shape change. It was concluded that the platelets in these samples had most of the morphologic integrity intact; however, the functional integrity appeared to be lost.

5. Fixation in paraformaldehyde (2 %) with 1 M trehalose.

After thawing and resuspension in UNISOL, these samples showed little clumping and 30 % - 50 % of the platelets had normal discoid morphology. Size distribution also demonstrated normal distribution indicating maintenance of morphologic integrity with no fragmentation or swollen (balloon) platelets. Also, when resuspended and tested in CPD-plasma, the platelets showed some functional response to hypotonic shock and shape change. It was concluded that the platelets in these samples had most of the morphologic integrity intact, and that also some of the functional integrity appeared to be present.

LYOPHILIZED RED CELLS.

1. Use of various concentrations of trehalose, sucrose, or KCl with ETOH.

0.25-1.00 M trehalose or sucrose and 25-100 mM KCl were used in these studies. Microscopic examination of the samples after thawing was performed. Of the RBC only 0.1 % - 1.0 % were intact and of those 50 % were crenated. No effect was found with various concentrations of trehalose and KCl. It was concluded that the RBC in these samples had lost their morphologic integrity and were not suitable for transfusion and also, that trehalose, sucrose, KCl and ETOH did not provide any protective effect.

2. Fixation in glutaraldehyde using autologous CPD-plasma.

Microscopic examination of the samples after thawing showed that the cells were intact with 90 % - 95 % showing normal discoid shape; however, some clumping was present. Functional deformability testing of these RBCs was performed by measurement of the time it took a 5 mL sample of the RBC resuspended in ADSOL (commercially available RBC additive solution) to run through a 20 μ m pore size filter as compared to a fresh sample of whole blood diluted to a similar Hct.

The fixed sample took 11 min and 27 sec compared to 2 min and 50 sec for the fresh sample, thus suggesting lack of deformability of the fixed cells.

It was concluded that the RBC in these samples had maintained their morphologic integrity; however, they had lost their deformability and were not suitable for transfusion.

3. Fixation in KMnO_4 with sucrose.

All the samples were thawed and resuspended in UNISOL. All samples showed less than 5% intact red cells. It was concluded that the RBC in these samples had lost their morphologic integrity and were not suitable for transfusion and that fixation with KMnO_4 did not provide any protective effect.

SUMMARY AND CONCLUSIONS

PLATELETS.

A very promising finding in these studies is that lightly paraformaldehyde-fixed platelets appear to tolerate lyophilization, and, after resuspension in citrated plasma, demonstrated well-preserved morphology and maintenance of functional properties such as adhesion and a shape change response to the agonist ADP. This suggested that these platelets were still viable with intact metabolic and functional properties.

Platelets fixed in a low concentration of KMnO_4 with trehalose/TKIS and human serum albumin also appear to tolerate lyophilization well. These samples showed that 30 % - 50 % of the platelets had normal discoid morphology although there was no functional response with hypotonic shock and shape change.

RED CELLS.

RBC fixed in glutaraldehyde appear to tolerate lyophilization. However, these fixed cells showed lack of deformability as measured by filtration and are not suitable for transfusion. Rehydration of lyophilized red cell samples where KMnO_4 was used as fixative in the presence of trehalose or sucrose did not provide any protective effect since the rehydrated samples contained less than five percent intact red cells.

SUMMARY:

Freeze-dried human and animal platelets have been evaluated in integrity and function studies and shown to be potentially useful as a hemostatic resource with a much longer shelf-life than current platelet concentrates. The next set of objectives will focus on acquiring further data in vivo and in vitro to develop a protocol for preclinical human trials. At this stage of development, we have partially characterized at least two successful methods to stabilize platelets for lyophilization without obliterating function and responsiveness. The continuation of this work will determine the circulatory lifespan of these preparations and their regulation of the fibrinolytic system in wound healing. Adaptation of the preparation technique(s) to sterile processing and large scale unit doses will accelerate our progress towards a safe and efficacious transfusion resource.